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Mast cell numbers in incisional wounds in rat skin as a function of distance, time and treatment

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SUMMARY

The dynamic changes in skin mast cell (MC) numbers around incised wounds were studied, using experimental designs amenable to multiple analyses of variance. Sixty-four Wistar albino rats were shaved in the interscapular region, wounded or not wounded, and then killed 2 or 10 days later. During this period, the rats were exposed continually to a cold (2°C) or control (20°C) climate and treated daily over the shaved region with either tap water or a weak sulphuric acid (pH 3·5) solution. The MCs within five adjacent fields of the wound or the control reference and within the superficial and deep halves of the skin were counted (at × 400). The greatest decrease in MC numbers occurred within about 700 μm of the wound. Whereas the paucity of MCs within the wound region was evident at 2 days, near-normal levels were achieved by day 10. Cold exposure produced little effect, but MCs responded differently to the water and acid treatments as a function of distance and skin depth.

Several authors (Holczinger & Devenyi, 1954; Wichmann, 1955; Boyd & Smith, 1959; Reynolds *et al.*, 1963; Matsuda & Kitamura, 1981) have implicated the skin mast cell (MC) in wound healing. The disappearance and reappearance of these multipotent cells around incisions have been reported by Wichmann (1955) and by Boyd & Smith (1959).

Experiments in which MCs have been counted as a function of the distance from the wound (e.g. Holczinger & Devenyi, 1954) have contained sampling problems or have not been amenable to clear statistical analysis. No study has employed experimental designs that would allow the detection of possible interactions between post-wound time, various treatments and distance from the wound. The use of statistical procedures that simultaneously employ temporal variables and distances and depths within the skin could allow the detection of dynamic changes in MC numbers.

The present study was designed to determine the effects of incised wounds upon MC

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numbers within the two major levels of the skin as a function of distance and time. To further test the practical possibility that MC numbers in wounded skin might respond differently from MCs in non-wounded skin to environmental influences, two other treatments were selected: the pH of fluid applied to the wound and the ambient post-wounding temperature.

Both ambient temperature and the pH of adjacent tissue have been reported to influence wound healing. Humid conditions at the wound promote epithelialization (Osment, 1975; Sciubba *et al.*, 1978) while cold environments (2°–3°C) decrease the rate of healing (Filston & Vennes, 1968) and warm temperatures (30°C) facilitate healing (Cuthbertson & Tilstone, 1967). The pH of wounded tissue is known to shift from acidic to basic levels during the repair process (Reynolds *et al.*, 1963). Granulocytes and fibroblasts are very sensitive to lower ambient pH (Peacock & Van Winckle, 1970; Silver, 1980). In fact, pH gradients have been suggested as a major stimulus for wound repair (Silver, 1980).

METHODS

Sixty-four male albino Wistar rats obtained from Woodlyn Laboratories (Guelph, Ontario) were used as subjects. In experiment I (thirty-two animals), the subjects were 60 days of age (mean wt 268 ± s.d. 11·7 g) while in experiment II (thirty-two animals), the subjects were 90 days of age (mean wt 350 ± s.d. 20 g) at the beginning of the treatments.

From 1 week before the experiment until its end, all rats were housed singly in metal wire cages. Purina rat chow and water were available *ad libitum*. The following colony conditions existed: temperature 23° ± 1°C and LD cycle 12:12.

On the day of the experiments, the animals were randomly assigned according to a 2 × 2 × 2 factorial design (three-way analyses of variance). For both experiments I and II, the animals were allocated to one of two wound treatments (wound-control versus wound) and to one of two kill times (2 days versus 10 days). The third treatment was different for the two experiments. In experiment I, the animals were allocated to either the tap water (control) or acidic pH condition while in experiment II the rats were assigned to either the control or cold environment.

On the day of the wound or wound-control treatments, each animal was injected i.p. with 40 mg/kg of sodium pentobarbital (Somnitol) and 0·4 cc of atropine sulphate. Rectangular-shaped areas of about 3·5 cm by 2·5 cm were produced in the interscapular regions of all animals by an electric razor. Animals in the control-wound group were stroked with a blunt instrument over the shaved skin. For the wound groups, 1·5 cm length incisions were made (through the skin to the underlying fascia) at an angle perpendicular to the long axis of the rat by a small pair of dissection scissors (experiment I) or a razor blade (experiment II). The wound was held closed manually or with tape for about 15 min before the animals were placed in plastic recuperation cages within the surgery room.

In experiment I, the animals were returned to the home cages in the colony. Half the numbers of rats from each of the treatment conditions were treated daily (starting 15 min after shaving) with either tap water (pH = 6·4) or weakly acidic dilute solutions of sulphuric acid (pH = 3·5); these solutions were applied at room temperature and made fresh every third day. The fluids were administered with saturated (dripping) cotton puffs applied over the shaved areas for 15 s so that the areas were well soaked. Fresh puffs were used for each animal and the order in which the solutions were applied was altered daily.

In experiment II, 6 h after the injection of the anaesthetic, animals were taken to a room containing a walk-in environmental chamber. Half the animals were maintained in the chamber (2° ± 1°C) while the other animals (temperature controls) were placed outside (20° ± 1°C).

Animals were maintained in similar single wire cages and given food and water *ad libitum*. Both groups were given a LD 12:12 regime similar to the initial colony conditions.

Either 2 or 10 days after the wound (or no-wound) treatments, each rat was weighed and killed by chloroform. Because of the hair growth in the day 10 groups, the area around the wound was shaved carefully. Each shaved area was then carefully removed and placed immediately between two glass slides in a tray of E:F:A (90 parts 80% ethanol:5 parts 30% formaldhyde:5 parts glacial acetic acid) in order to reduce curling. After a period of 10 min, the tissues were then placed into a separate jar of E:F:A for 24 h. Following dehydration, clearing and infiltration (Persinger, 1980; 1981) tissues were embedded in paraffin blocks. The blocks were sectioned at 6 μm in a plane perpendicular to the surface of the skin (and to the long axis of the incision). Five serial sections for every 100 sections were stained with toluidine blue for mast cells. In experiment I, other sections were stained with fuchsin degenerative and Verhoeff elastin stains (Humason, 1972).

Mast cells, as defined by both metachromasia and morphology, were counted at $\times$ 400 within the five adjacent fields from the wound and within the two adjacent fields of the upper and lower halves of the skin ($5 \times 2 = 10$ fields). Optimal sections (minimal histological artifacts) at the level that most demonstrated the wound were selected. As an extra precaution (in expermiment I) since there was some overlap between the upper and lower fields of some sections, the average dermal depth of each tissue (section) was determined by a Trisimplex projector for later analyses of covariance.

In experiment I, the eight fields on one side and the eight fields on the other side of a wound (which was aligned in the middle of the two central fields) were counted and averaged for MCs. In experiment II, the ten fields were counted for only one side of the wound, but two separate sections (separated by 100 μm) were counted and averaged. For the control (no-wound) tissues, comparable regions in the middle (experiment I) or the edge (experiment II) of the tissue were selected as the central two fields. All tissues ($n = 32$) were sectioned, stained and counted for MCs. One tissue was lost in experiment II ($n = 31$).

Five-way analyses of variance with two factors repeated (two wound conditions, two kill days, two specific treatments, five fields from the wound and two fields of skin thickness) were completed by BMD statistical software using a DEC2020 System computer. Non-repeated measure analyses and checks for numerical properties of the data were verified using SPSS software.

Since some of the MC populations per field exceeded skewness values of 1·0, square roots of the MC numbers were also computed for both experiments. These data transformations reduced all skewness values to less than $\pm$ 0·9. Five way analyses of variance plus covariance for either body weight, change in body weight, surgical sequence, tier in the home cages, tier in the experimental cages, or thickness of tissue (experiment I only) were completed for the absolute and square root data.

RESULTS

The means and standard deviations for mean MC numbers/field for the three main effects and the skin depth are presented in Table 1. Five-way analyses of variance with two factors repeated (skin depth and distance) indicated no significant main effects for either the wound, time or special treatment conditions. However in both experiment I (Fs = 107·79, 153·89, d.f. = 1·24; the second F values refer to analyses of square roots of MC numbers/field), and experiment II

TABLE 1. Means and standard deviations (±) for mean numbers of mast cells per field for wound, time, environmental treatments and skin depth

Experiment I			Experiment II	
		Wound		
Control	Wound		Control	Wound
12·4±4·5	10·9±2·8		4·4±1·1	3·9±1·2
		Time		
2 Day	10 Day		2 Day	10 Day
11·5±4·6	11·8±2·9		4·2±0·9	4·2±1·4
		Environment		
H_2O	Acidic		Warm	Cold
11·6±3·5	11·7±4·2		3·8±0·8	4·5±1·4
		Skin depth		
Upper skin	Lower skin		Upper skin	Lower skin
7·1±2·7*	16·2±5·8†		2·4±1·2*	6·0±1·7†

* Versus† = $P < 0.001$.

(Fs = 125·91, 158·80, d.f. = 1·23) the upper skin had significantly ($P < 0.001$) fewer MCs/field than the lower tissue.

Both experiments displayed significant ($P < 0.01$) distance by wound interactions (Fs = 7·44, 14·56, d.f. = 4·96 for experiment I and Fs = 3·97, 6·45, d.f. = 4·92 for experiment II). As can be seen in Fig. 1, this interaction was due to the smaller number of MCs in the vicinity of the wound site. Interestingly, both experiments showed significant ($P < 0.05$) depth by distance interactions (Fs = 4·26, 3·00, d.f. = 4·96 for experiment I and Fs = 3·03, 6·56, d.f. = 4·92 for experiment II). *A posteriori* analyses indicated this effect was due primarily to the wounded animals (Fig. 2).

Significant wound by time (Fs = 7·05, 11·00, d.f. = 1·24), distance by wound by time (Fs = 3·12, 8·34, d.f. = 4·96), depth by distance by time (Fs = 2·14, 4·02, d.f. = 4·96) and a depth by distance by application (Fs = 3·80, 2·56, d.f. = 4·96) were also apparent in experiment I only. The first two interactions can be surmised from Fig. 3 and are due primarily to the return of MC numbers in the wound region to control values for the 10-day but not the 2-day rats. The only significant modification of MC numbers by environmental treatments, in this case, the application of either tap water or weak sulphuric acid to the wound is shown in Fig. 4. Experiment II only showed a marginally significant ($P = 0.05$) depth by wound interaction (Fs = 3·32, 5·87, d.f. = 1·23). This was due primarily to the smaller number of cells in the upper skin only (see Fig. 2) of the wounded animals relative to the non-wounded animals.

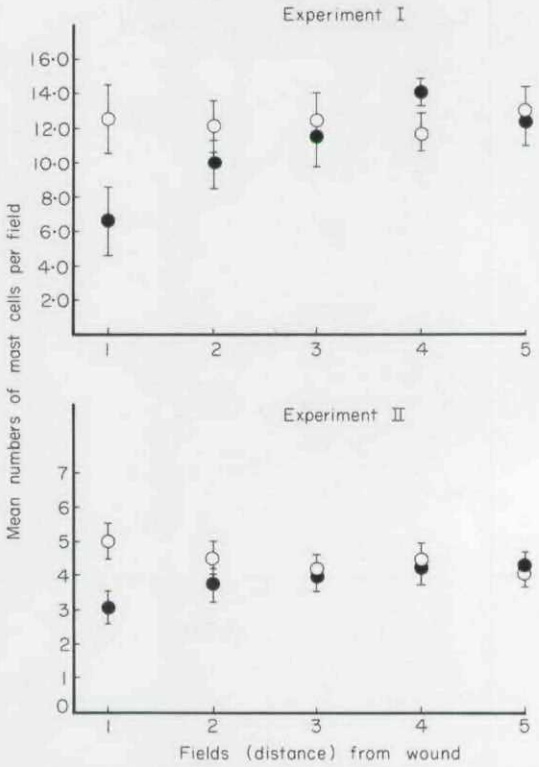

FIGURE 1. Mean numbers of mast cells/field for wounded (●) and non-wounded (○) skin as a function of the distance from the wound (or comparable reference point) for experiments I and II. Vertical bars indicate standard errors of the mean.

Analyses of co-variance for body weight, changes in body weight during the experiment or position in the housing racks did not alter the F values or produce any significant coefficients. Co-variance for thickness of the sections (for experiment I only) did not alter the F values in any significant manner.

However, there were significant ($P < 0{\cdot}001$) differences in section thickness between the two kill times. Three-way analyses of variance (time by wound by treatment) demonstrated ($F = 17{\cdot}01$, d.f. $= 1{\cdot}24$) that the skins of the animals killed at 10 days ($1{\cdot}0 \pm 0{\cdot}2$ mm) were thicker than the animals killed at 2 days ($0{\cdot}8 \pm 0{\cdot}2$ mm). However, there were no significant wound or treatment effects or interactions (all Fs $\leq 1{\cdot}00$). The means and standard deviations for section thickness of the no-wound 2 day, wound 2 day, no-wound 10 day and wound 10 day groups were $0{\cdot}7 \pm 0{\cdot}1$, $0{\cdot}9 \pm 0{\cdot}2$, $1{\cdot}1 \pm 0{\cdot}2$, and $1{\cdot}0 \pm 0{\cdot}2$ mm, respectively.

Qualitative evaluation of the wound region at × 400 demonstrated features within the normal range of healing. For example, in experiment I only, three of the eight wounded rats killed after 2 days had failed to complete epithelialization; all wounded rats killed after 10 days displayed this feature. Pinkish lines and networks were noted in the fuchsin-stained sections for the 2 day wounded rats but not in the 10 day wounded rats. More material around the wound was noted by the collagen stain in the 10 day than in the 2 day tissues.

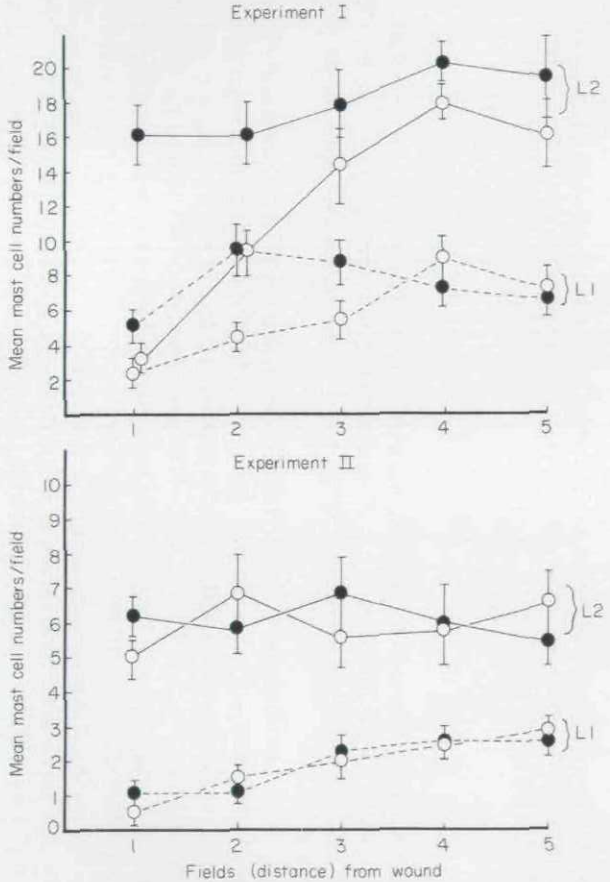

FIGURE 2. Mean numbers of mast cells/field in the upper (– – – –) and lower (———) portions of the skin as a function of distance from the wound at either 2 (○) or 10 (●) days after the incision for wounded rats only. Vertical bars indicate standard errors of the mean.

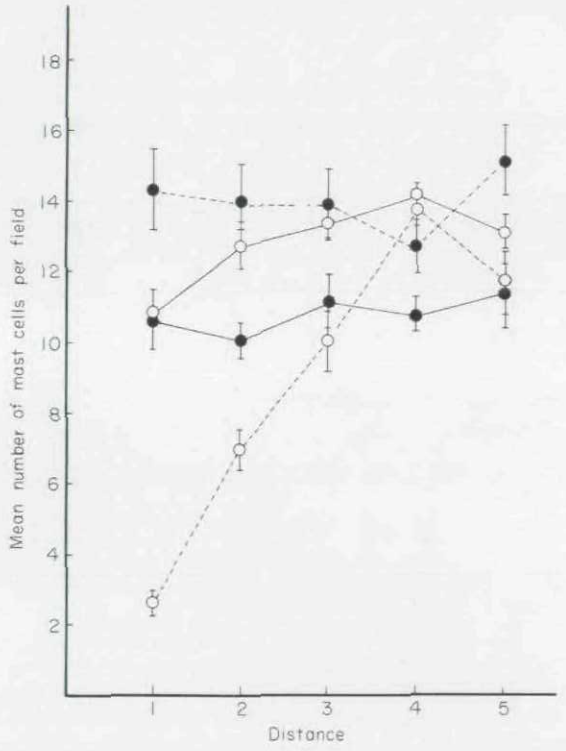

FIGURE 3. Mean numbers of mast cells/field as a function of distance for the non-wounded and wound groups on day 2 or day 10. Vertical bars are standard errors of the mean. Wound day 2 (○– – –○; wound day 10 (○———○); non-wound day 2 (●– – –●); non-wound day 10 (●———●).

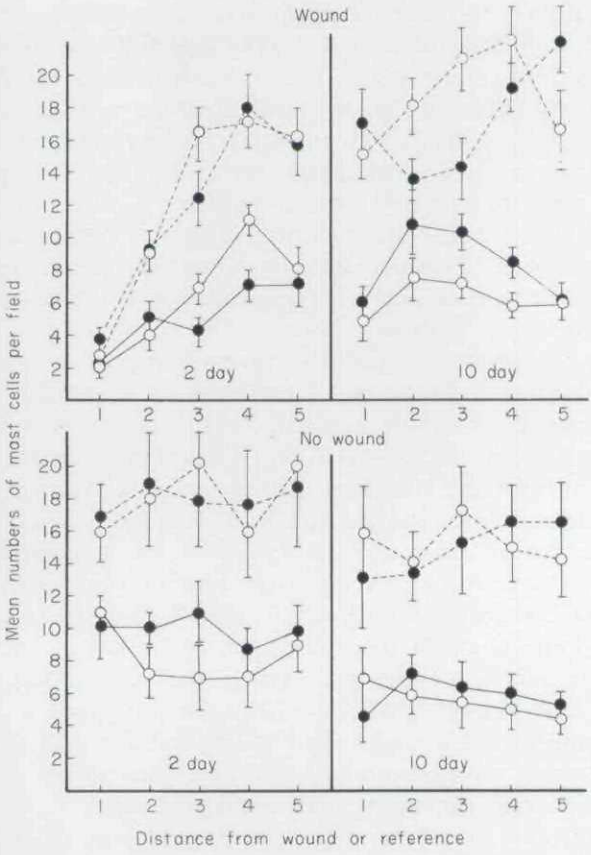

FIGURE 4. Mean numbers of mast cells/field in the upper (———) and lower (– – – –) levels of the skin for the 2 and 10 day animals as a function of distance from the wound or reference site and tap water (pH = 6·4) or weak sulphuric acid (pH = 3·5) treatments. Vertical bars indicate standard errors of the mean. pH = 6·4 (○); pH = 3·7 (●).

DISCUSSION

The present studies confirm several previous reports. Firstly, as noted by both Holczinger & Devenyi (1954) and Wilhelm, Yong & Watkins (1978), clear layer differences in MC numbers were evident. In both experiments I and II, the lower level, comparable with the dermis and hypodermis of the rat, contained about twice as many mast cells as the upper level. Estimates of mean cell density in this study ranged from 5000 to 10,000 MCs/mm^3; Wilhelm *et al.* (1978) reported a mean of about 7000 MCs/mm^3 in the skin of 60–80 day old rats.

Secondly, both the distance and time of MC changes support previous investigations. Skin MC numbers were very low (even zero in some cases) within 240 μm (radius of the microscopic field) 2 days after the wound but had returned to control group values by day 10. Boyd & Smith (1959) also noted an absence of MCs within 300 μm of the wound after 5 days of healing while Holczinger & Devenyi (1954) and Wichmann (1955) reported that the initial decrease in MCs near the wound was followed by an increase that peaked about 8–10 days later.

Distance from the wound is clearly important with respect to MC numbers. In both

experiments I and II, the greatest decrease in MC numbers occurred within the first two fields or (240 + 480 μm) 0·72 mm from the wound. No obvious differences in MC numbers existed beyond this distance, at least up to five fields or 2·1 mm. However since in experiment I the 10-day-old wounded skin still had fewer MCs than non-wounded controls, low-level decrements in MC numbers may be apparent only if still greater distances from the wound are evaluated.

The wounded regions in the present studies are comparable with previous reports. For example, the pinkish network noted by the fuchsin stains had been described by Ordman & Gilman (1966) for rat skin 2 days after wounding. This line, which has been described as a collagen precursor, was not noted in the 10 day rats. Since collagen synthesis is assumed to begin about day 4-5 after wounding (Dunphy, 1974) the collagen synthesis would be expected to be well into the proliferative stage in these animals.

Certainly the major discrepancy between the two experiments was the mean MC numbers/field. Although contributions from the application of fluids to the skin and changes with age cannot be totally excluded, the most likely major source of variance was inter-observer (criterion) differences. Whereas MC counts in experiment I involved any intact metachromatic cell with the appropriate form, the MC counts in experiment II required the presence of all or a portion of the cell nucleus. Such a criterion difference would have resulted in smaller numbers of MC counts in the second experiment; the results support this suggestion. Inter-experimenter reliability measures were not completed in this study; however (Cronbach) alpha coefficients for comparable MC measurements in other experiments have been shown to exceed 0·98.

The relatively small effects of pH treatment upon MC numbers and distributions within wounded tissues were not surprising considering the short duration of the treatment application. However, the lack of significant effect from prolonged cold room exposure was surprising. If lower temperatures retard wound healing and if wound healing is related to MC distributions, then certainly some deviation should have been noted.

Our contention that central effects generated by the cold exposure would contribute to MC differences around the wound was not supported. Perhaps more peripheral factors contribute to MC density. One possibility compatible with both the MC and wound healing literature would involve the local temperature evaluation around wounded tissue (Cuthbertson & Tilstone, 1966). The facilitation of wound healing in animals exposed to higher ambient temperatures could be related to the enhanced blood flow capillary invasion around the wounded area. Considering the frequent correlation between MC numbers and capillarization, MC numbers would be expected to proliferate around wounds of animals exposed to local elevations in temperature.

Mast cells in wounded skin appear to be very mobile. Although gross enumeration of MCs and simple t-test analyses have given first approximations of MC changes around wound sites, more sophisticated statistical and analytical techniques may be required to ascertain the dynamic profiles of these cells within the skin. Multiple analyses of variance designs employing several time intervals and repeated measures (as a function of wound distance) may be helpful tools. As demonstrated, these methods can also detect weak but potentially significant alterations in MC numbers from environmental treatments.

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